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(54) HIV peptide conjugated via anionic spacer to protein

(57) Peptide-protein conjugate vaccines having anionic spacers for connecting the peptide and protein moieties are water-soluble and may be used for immunizing mammals. A process for making such conjugates includes the option of using a detergent to maintain solubility during conjugation. The conjugate specifically comprises the outer membrane protein complex of *Neisseria meningitidis* b as the protein carrier and a principal neutralising determinant of the human immunodeficiency virus as the peptidyl epitope linked by the anionic spacer.

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10 TITLE OF THE INVENTION  
PEPTIDE-PROTEIN CONJUGATE VACCINES HAVING ANIONIC  
SPACERS

BACKGROUND OF THE INVENTION

15 It is known that many epitopes, in order to  
raise useful levels of immune responses in a mammal,  
must be conjugated to an immune enhancer. This is  
true of small peptides and other haptenic epitopes.  
One solution for peptide epitopes is to conjugate  
20 these to an immunogenic carrier protein such as  
pertussis toxoid, diphtheria toxoid, keyhole limpet  
hemocyanin, bovine serum albumin, human serum  
albumin, or the outer membrane protein complex (OMPC)  
of certain bacteria. A particularly preferred  
25 immunogenic carrier is the OMPC from Neisseria  
meningitidis. This carrier may be prepared, for

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example, according to the process of Helting as disclosed in U.S. Patent 4,271,147.

Polysaccharide-OMPC conjugates were disclosed in U.S. Patent 4,695,624. Many OMPC-peptide conjugates have since been produced and claimed in U.S. Patent applications 07/362,179 (OMPC-peptide conjugates), 07/555,558 (OMPC-polysaccharide-peptide conjugates), 07/555,966 (OMPC linked by a first linker to an anion and by a second linker to a peptide).

A conjugate vaccine, in order to be injectable, must be soluble or form colloidal suspensions. Some peptide/protein epitopes add a net positive charge to the carrier protein (due to Arg or other basic amino acids) through or after conjugation, causing (irreversible) precipitation of the conjugate. OMPC generally appears to be more sensitive to charge balance than other commonly used carrier proteins such as tetanus toxoid or bovine serum albumin. Control experiments have indicated that insolubility of a conjugate is due to addition of positive charge to OMPC. This invention incorporates negative charge into the tether connecting OMPC to peptide epitopes as an effective means of neutralizing this addition of positive charge.

This invention is particularly applicable to conjugates prepared with OMPC and human immunodeficiency virus (HIV) principal neutralizing determinant (PND) peptides which generally have one or more net positive charges per peptide molecule. Naturally, however, other carrier proteins, or other

peptide epitope combinations may result in similar solubility problems, and this invention would be obviously applicable to those systems.

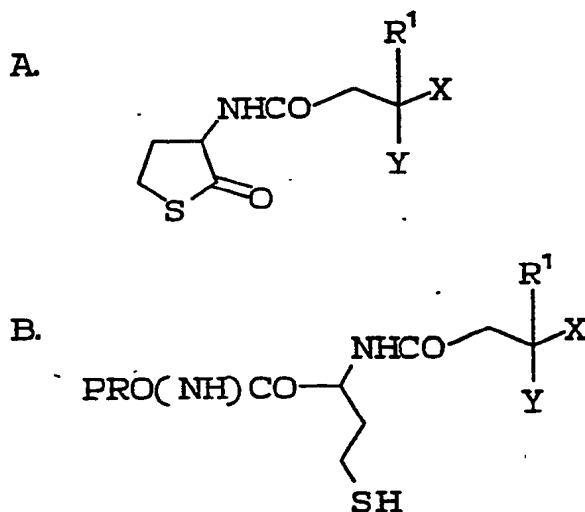
Conjugate vaccines with poor solubility may produce poor immune responses. Usually, insolubility is an indication of high epitope incorporation. Reduction in the level of incorporation of peptide epitope into the conjugate to increase solubility is self-defeating since the amount of the desired immunological determinant is thereby also decreased. Incorporation of an anionic spacer offsets positive charge in the peptide epitope, rendering the conjugate soluble. Klotz, I. M. and Heiney, R.E. [J.Am. Chem. Soc. 81, 3802-3803, (1959)] reported on the use of S-acetylmercaptosuccinic anhydride to thiolate proteins. However, the product of that work did not result in the production of an easily quantifiable spacer (mercaptosuccinyl), and only introduced a single negative charge. The anionic spacers disclosed in this invention overcome these limitations, and are new in conjugate constructs.

Insoluble OMPC conjugates, and insoluble vaccines have diminished utility. This invention provides a new solution to the problem of conjugate preparation, while allowing for high peptide epitope loading on immunogenic protein carriers such as OMPC.

#### SUMMARY OF THE INVENTION

A peptide-protein conjugate, wherein the protein is an immune enhancer and the peptide has at least one epitope against which immune responses in a mammal are desired, is prepared by conjugating the peptide and protein through a nonmacromolecular, anionic spacer or tether.

In a preferred embodiment of the invention, a compound of the type A is prepared which is susceptible to nucleophilic attack by amines on a protein carrier, PRO, to form a thiol-containing spacer molecule B, attached to PRO:



wherein:

PRO is the immunogenic protein;

R<sup>1</sup> is hydrogen, or lower alkyl;

(i) X is:

(a) -PO<sub>3</sub><sup>=</sup>,

(b) -SO<sub>3</sub><sup>-</sup>, or

(c) -CO<sub>2</sub>H; and

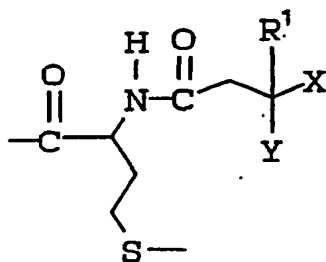
Y is hydrogen; or

(ii) both X and Y are -CH<sub>2</sub>CO<sub>2</sub>H, or

(iii) X is CO<sub>2</sub>H and Y is -CH<sub>2</sub>CO<sub>2</sub>H.

A thiol reactive peptide, such as a bromoacetylated peptide or a maleimidated peptide, is then reacted with the anionic, thiolated protein to form the conjugate product of the invention.

Thus, in a preferred embodiment of the invention, the conjugate comprises a spacer of formula:



wherein the free carbonyl on the left is bonded to OMPC and the sulfur is bonded to an HIV principal neutralizing determinant, PND;  
R<sup>1</sup> is hydrogen or a lower alkyl of 1 to 4 carbons;

(i) X is:

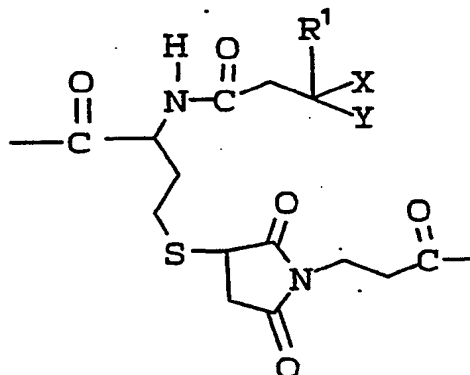
- (a)  $-\text{PO}_3^-$ ,
- (b)  $-\text{SO}_3^-$ , or
- (c)  $-\text{CO}_2\text{H}$ ; and

Y is hydrogen; or

(ii) both X and Y are  $-\text{CH}_2\text{CO}_2\text{H}$ , or

(iii) X is  $\text{CO}_2\text{H}$  and Y is  $-\text{CH}_2\text{CO}_2\text{H}$ .

Where a maleimidated peptide is used, the spacer may be further defined as having the formula:



wherein PRO is bonded to the carbonyl on the left, the HIV PND is bonded to the carbonyl on the right, and R<sup>1</sup>, X and Y are as defined above. In the process of this invention, where a hydrophobic peptide is being conjugated, the process utilizes a detergent to enhance solubility of the conjugate.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention is a conjugate of an immunogenic protein and peptides displaying at least one epitope against which an immune response is desired. The peptide and immunogenic protein are tethered by a non-macromolecular anionic spacer (molecular weight below about 1000 daltons).

Immunogenic proteins are known in the art and may be pertussis toxoid, diphtheria toxoid, bovine serum albumin (BSA), human serum albumin (HSA),

Keyhole limpet hemocyanin (KLH) or the outer membrane protein complex (OMPC) of certain bacteria. In a preferred embodiment of this invention, the immunogenic protein is the OMPC from Neisseria meningitidis b. One method for obtaining this protein is according to the method of Helting, disclosed in U.S. Patent 4,271,147. In addition, OMPC subunit proteins may be used in this invention, or recombinant or synthetic portions thereof may be used to provide the immune enhancing function.

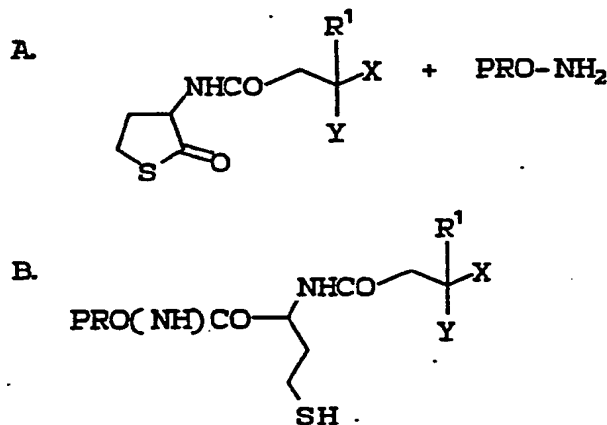
The peptide portion of the conjugate may be any peptide, synthetic, recombinant, or natural, exhibiting at least one epitope against which an immune response is desired. The invention is particularly applicable to peptides which are not very immunogenic on their own, and which have a net positive charge at physiological pH. By coupling the peptide to the immunogenic carrier protein described above, through a non-macromolecular anionic spacer, an immunogenic complex is provided which is soluble in aqueous media.

Of the peptides that are of current interest, those peptides bearing a principal neutralizing determinant (PND) of the human immunodeficiency virus (HIV) are particularly preferred for conjugation according to this invention. Thus, peptides bearing the trimer Gly Pro Gly, the tetramer Gly Pro Gly Arg [Sequence. Id:1:], and extensions thereof, whether in a linear or cyclic configuration, are preferred in this invention. Peptides of this type are described further below.



The spacer is a molecular chain for linking the peptide and immune enhancing moieties to each other. Preferably, the spacer exhibits between about one and five negative charges at physiological pH. It is also preferred that the spacer be hydrolytically stable such that upon hydrolysis of the peptide or immune enhancing moieties for amino-acid analysis, the hydrolytically stable spacer may also be quantitated as described in U.S. Patent 4,695,624 and in Marburg et. al., J.A.C.S. 108, 5282-5287 (1986).

In a preferred embodiment of the invention. A compound of the type A is prepared which is susceptible to nucleophilic attack by amines on a protein carrier, PRO, to form a thiol-containing spacer molecule B, attached to PRO:



wherein:

PRO is an immunogenic protein;

R<sup>1</sup> is hydrogen or lower alkyl of between 1 and 4 carbons;

(i) X is:

- (a)  $-\text{PO}_3^-$ ,
- (b)  $-\text{SO}_3^-$ , or
- (c)  $-\text{CO}_2\text{H}$ ; and

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Y is hydrogen; or

(ii) both X and Y are  $-\text{CH}_2\text{CO}_2\text{H}$ , or

10

(iii) X is  $\text{CO}_2\text{H}$  and Y is  $-\text{CH}_2\text{CO}_2\text{H}$ .

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A thiol reactive peptide, such as a bromoacetylated peptide or a maleimidated peptide is then reacted with the anionic, thiolated protein to form the conjugate product of the invention.

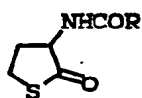
Thus, according to a preferred embodiment of this invention, a conjugate is prepared as shown in Scheme 1:

#### SCHEME 1

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#### THIOLATION AND PEPTIDE CONJUGATION OF OMPC

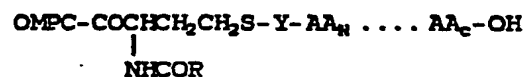
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+ OMPC



A



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AA<sub>N</sub> = N-terminal amino acid residue and AA<sub>C</sub> =  
C-terminal amino acid residue; X = electrophilic group  
such as bromoacetyl or 3-(N-maleimido)propionyl; Y =  
methylenecarbonyl or 3-(N-succinimid-3-yl)propionyl;  
5 R = -(CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>H; -(CH<sub>2</sub>)<sub>2</sub>PO<sub>3</sub>H; -(CH<sub>2</sub>)<sub>2</sub>SO<sub>3</sub>H;  
-CH<sub>2</sub>CH(CH<sub>2</sub>CO<sub>2</sub>H)<sub>2</sub>; -CH<sub>2</sub>C(CH<sub>3</sub>)(CH<sub>2</sub>CO<sub>2</sub>H)<sub>2</sub>.

The syntheses of the various anionic  
thiolactones, shown in Scheme 2, were effected either  
10 by direct acylation of homocysteine thiolactone, 2,  
or by conversion to the bromopropionyl derivative, 5,  
followed by displacement of the bromine with the  
appropriate nucleophiles. The preparation of the  
phosphonic acid, 7, proceeded via the phosphono  
15 dimethyl ester, 6, which was demethylated with  
trimethylsilyl bromide according to Chakravarty *et*  
*al.* [Tetrahedron Lett. 28, 611-612 (1987)]:

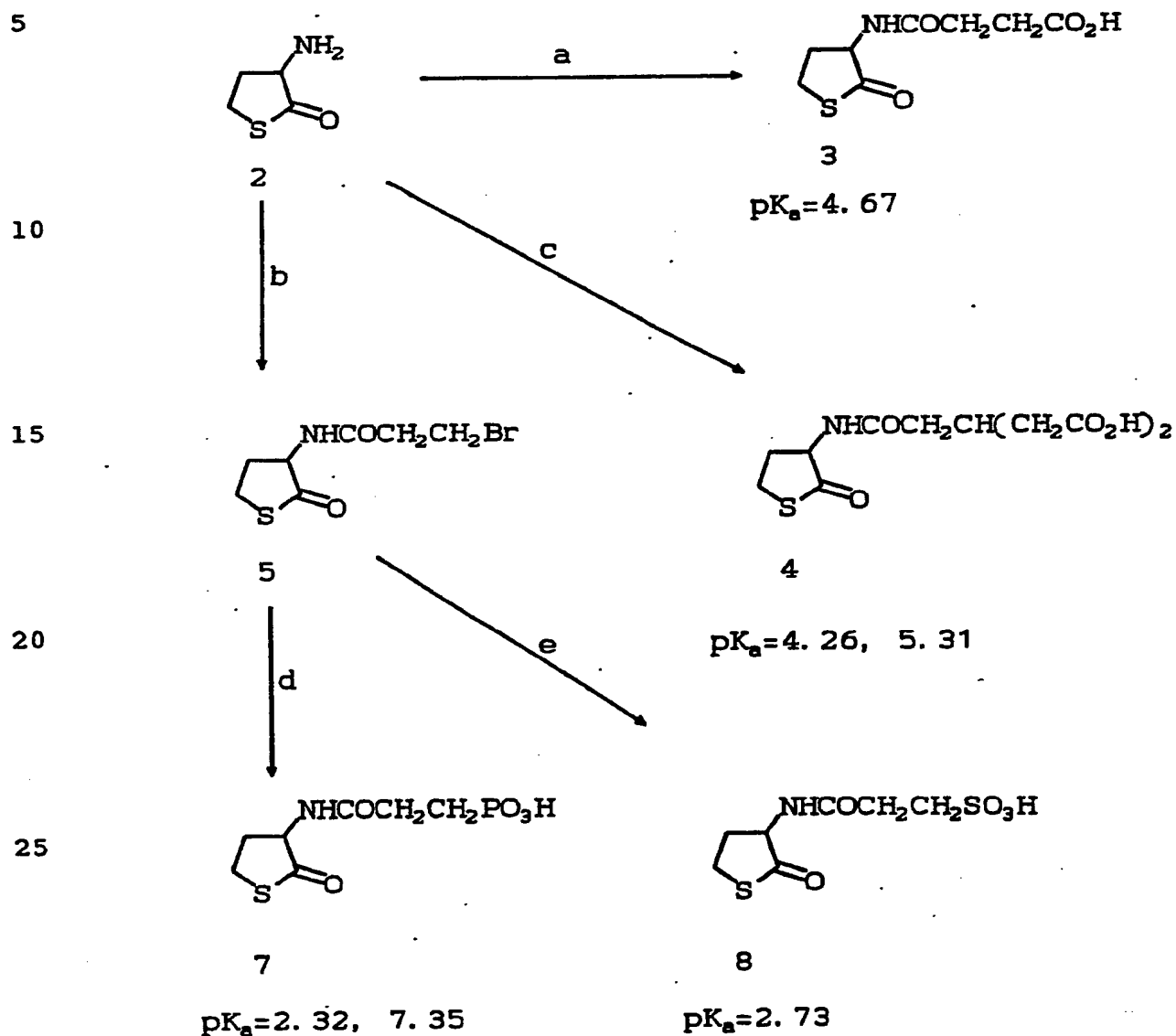
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SCHEME 2

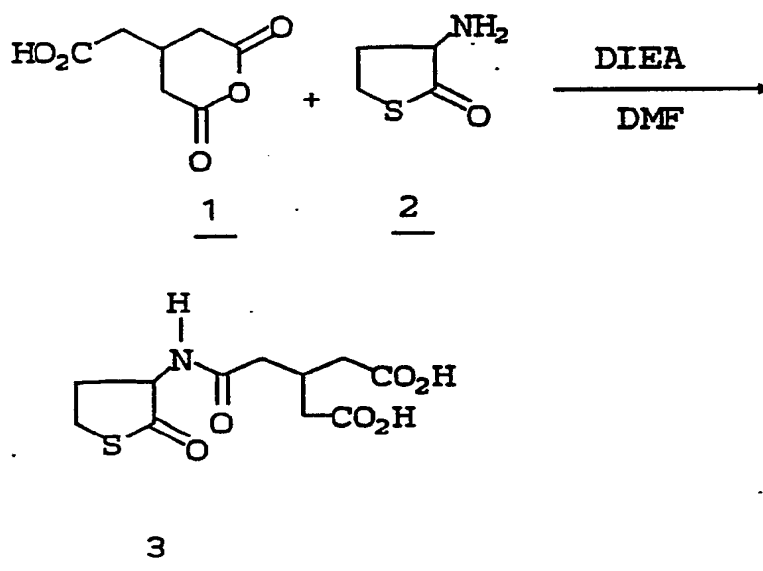
## SYNTHESIS OF ANIONIC THIOLACTONES



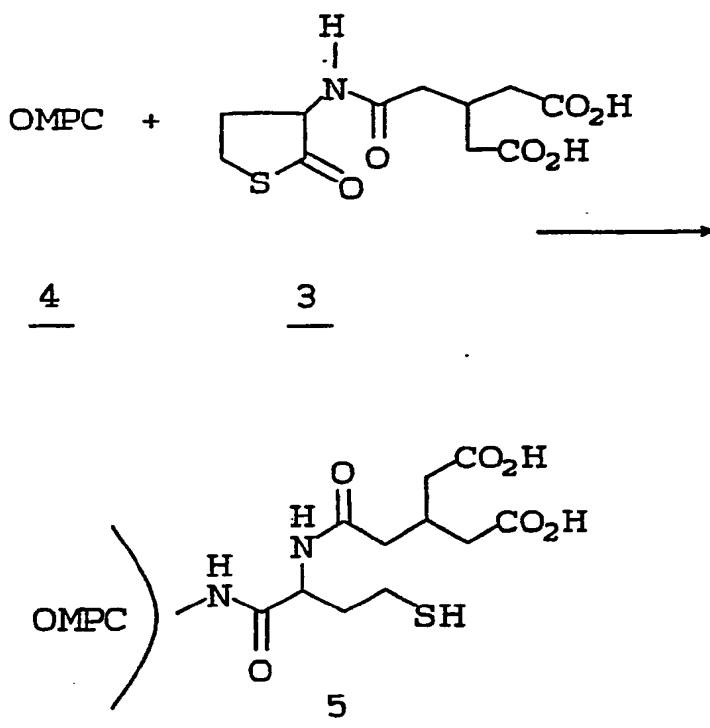
Reagents: (a) succinic anhydride; (b) bromopropionyl chloride; (c) carboxymethylglutaric anhydride; (d) trimethyl phosphite then trimethylsilyl bromide; (e) sodium bisulfite.

Thus, in one embodiment of the invention, 3-carboxymethylglutaryl-homocysteine thiolactone (CMGTL) 3 is prepared from carboxymethyl glutaric anhydride 1 and homocysteine thiolactone hydrochloride 2 according to Scheme 3:

SCHEME 3



wherein DMF is dimethylformamide, and DIEA is diisopropylethylamine. An immunogenic carrier protein such as OMPC 4 is then reacted with the CMGTL 3 to produce anionic thiolated OMPC 5 according to Scheme 4:

SCHEME 4

Although Scheme 4 shows the OMPC surface with one amine function having been derivatized, it is to be understood that as many amine functions as are available on the OMPC surface may be so derivatized. Multiple derivatizations in this manner provides an immune enhancer to which multiple peptidyl epitopes may be conjugated.

The role of a homocysteine thiolactone in the conjugation chemistry of OMPC involves acylation of the amino groups of OMPC to append a thiol group to the protein. This thiol group is then alkylated

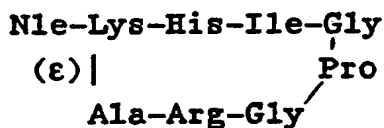
by an electrophile, such as a bromoacetyl group, which is incorporated into the molecule (i.e. peptide) to be conjugated. This is outlined in Scheme 1 above. The stable thioether formed in the process not only affords the covalent bond between OMPC and the ligand (peptide) but also, in appropriate cases, allows the degree of ligand binding to be evaluated by analysis of the newly-formed amino acid, S- Carboxymethylhomocysteine thiolactone. Other electrophiles such as malemidated peptide epitopes may also be used to alkylate the thiolated proteins of this invention in which case the degree of conjugation is measured by assay of a "marker" amino acid such as b-alanine or norleucine.

Since HIV peptides are available in limited quantities and express a variety of characteristic (e.g. hydrophobic groups) which could affect the solubility of product, bromoacetyl triarginine was chosen as a model peptide for initial experiments.

With complex peptide ligands the effect of the anionic spacers may not always result in complete solubility of the conjugate because the solubility of derivatized proteins is dependent on many factors. In this regard, one embodiment of the invention includes performing the conjugation reaction in the presence of a detergent. We discovered that when a conjugate which exhibited solubility problems, even when the peptide was conjugated using a  $-PO_3^-$  spacer, was prepared in the presence of deoxycholate, the conjugate was completely soluble. In this regard, any detergent which can be used without adverse reaction to a recipient may be expected to behave

similarly in ameliorating persistent solubility problems. A preferred conjugation medium, in accord with this discovery, is TED (0.1 M TrisHCl pH 8.5, 0.01 M EDTA, 0.5% deoxycholate). The conjugation can be run in this buffer, as it does not interfere with the conjugation chemistry, simply by pelleting and resuspending the thiloated OMPC in the buffer. In addition, the detergent may be removed by dialysis or any other buffer exchange step once the conjugate has been prepared, and the conjugate may be recovered for administration. Thus, the availability of these anionic thiolactones for use as linking molecules, and the disclosure of a conjugation process utilizing detergent, provides the possibility of changing the physical properties of varied types of macromolecules, affording fruitful conjugations and usable conjugate materials.

A synthetic peptide, such as cPND815, (Seq. ID: 2:)

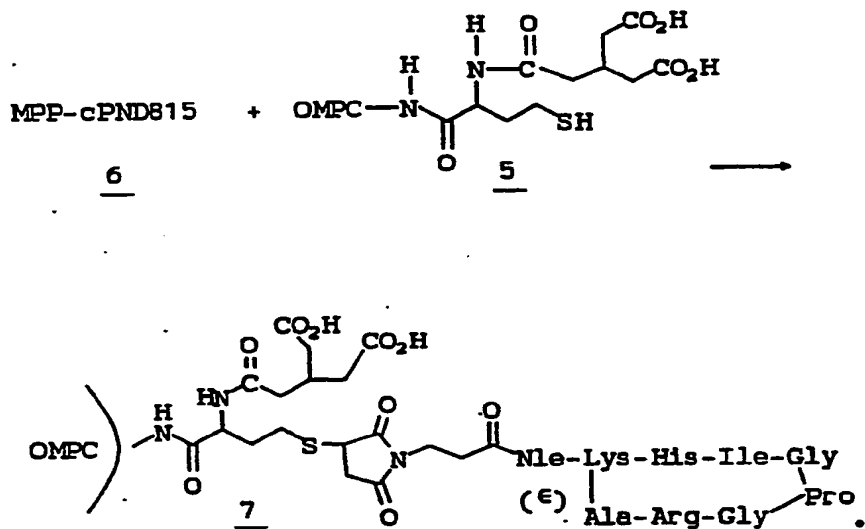


wherein the cycle is formed by an amide bond through the ( $\epsilon$ ) amino group of lysine to the carboxy-terminal alanine of the peptide, and the ( $\alpha$ ) amino group of lysine is bonded to norleucine, is derivatized so as to be reactive with the derivatized



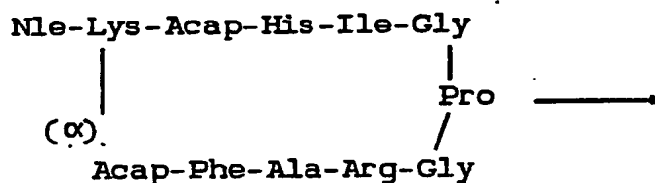
protein. This may be accomplished, for example, by maleiminating the peptide by treatment with maleimidopropionyloxysuccinimide or a similar reagent to produce maleimidopropionyl cPND815, MPP-cPND815 6. Alternatively, the peptide may be bromoacetylated with a reagent such as p-nitrophenyl bromoacetate. The bromoacetylated peptide may then be reacted with the thiolated protein.

A maleiminated peptide, such as MPP-cPND815 (seq. Id:2:) 6, is then reacted with the thiolated, anionic OMPC 5 to form a conjugate 7 of this invention, according to Scheme 6:

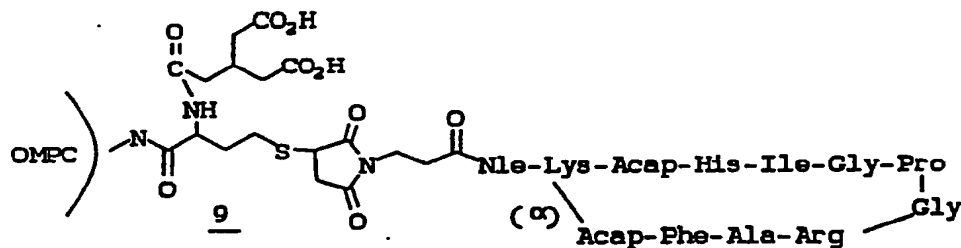
SCHEME 6

In a similar fashion, using MPP-cPND955 (Seq. ID: 3:), 8 the conjugate 9 is produced:

SCHEME 7

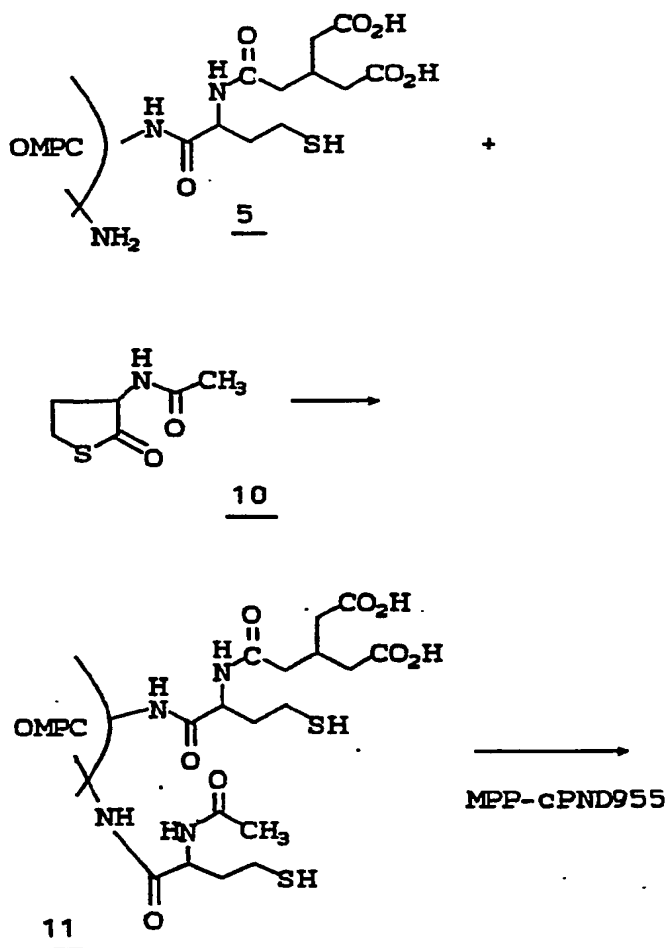


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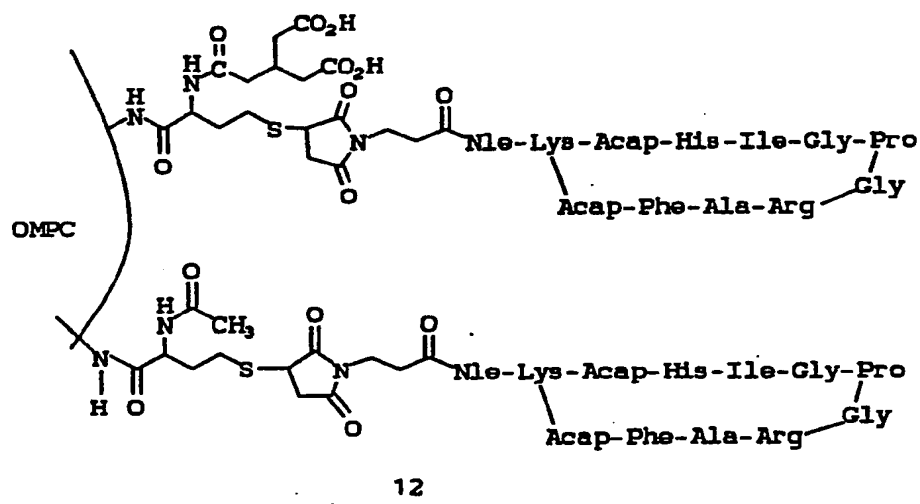


Naturally, onto each OMPC is appended more than one anionic spacer-peptide complex. The higher the titer of such peptidyl epitopes, the higher the expected immune response against the epitope being presented. In addition, it is to be understood that use of a mixture of different cPND's at this point would yield a conjugate protective against a broad variety of HIV isolates. Furthermore, anionic groups other than  $-\text{CO}_2\text{H}$ , such as  $-\text{PO}_3^-$  or  $-\text{SO}_3^-$ , or mixtures of these, is also be desirable.

As more and more anionic spacers are appended, however, the charge repulsion appears to inhibit saturation of reactive sites on OMPC. However, at this point neutral reagent, such as N-acetyl homocysteine thiolactone 10 may be added to the OMPC already saturated with anionic spacers, to give a highly substituted OMPC 11 which can then react with maleimidated peptides to give a highly loaded conjugate 12, according to Scheme 8:

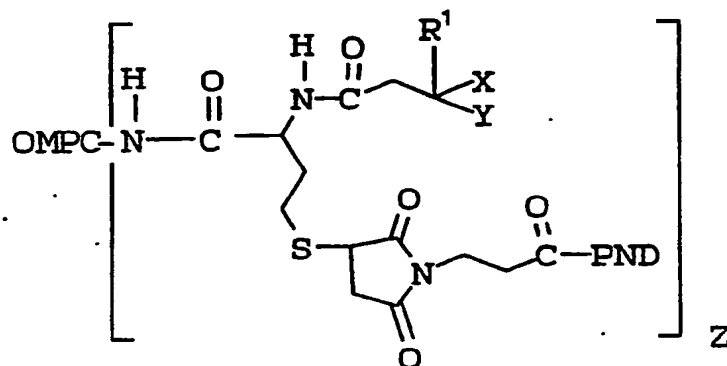
SCHEME 8

SCHEME 8 cont'd.



Thus, a conjugate of the type 12 comprising both a neutral and an anionic spacer naturally falls within the scope of this invention.

Hence, in one preferred embodiment of the invention, the conjugate immunogen has the formula:



wherein:

R<sup>1</sup> is hydrogen or lower alkyl of between 1-4  
carbons;

X and Y are as defined above; and

5 Z is  $1 \leq 6000$ , assuming a molecular weight for  
the OMPC complex of about  $40 \times 10^6$  daltons,  
and an average loading of about 75-100  
nmoles of peptide per milligram of OMPC.  
Preferably, Z is about 3000.

10 The PND may be a single species or a mixture  
of different species, such that immune responses  
directed at a broad range of HIV isolates is  
generated upon immunization. Any of a number of  
15 peptides, the primary sequence of which is suggested  
for Example, by LaRosa et. al., Science vol 249, p  
932-935, (1990), or by Rusche W0 90/03984 (1990) in  
linear or cyclic form, may be used. The cycle may be  
through a disulfide, amide, thioether or any other  
20 convenient ring closure means. Thus, according to  
this invention, the term HIV PND should be understood  
as representing any peptide capable of inducing an  
HIV neutralizing immune response. In particular,  
peptides having the sequence -Gly Pro Gly Arg- (Seq.  
25 Id:1:), and including up to a total of about 40 amino  
acids in a cyclic configuration, are preferred.

In addition to the specific peptides  
described above, other cyclic peptides have been  
conjugated according to this invention. These  
30 include:

## PEPTIDE SEQUENCE

cPND502 SEQ. ID:4

Lys Abu His Ile Ile Gly Pro Gly Arg Ala Phe

5

cPND535 SEQ. ID:5

Lys Abu Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala  
Phe Val Thr Ile Gly

10

cPND078 SEQ. ID:6

Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Cys

15

cPND402 SEQ. ID:7

Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile  
Gly Cys

20

cPND495 SEQ.ID:10

Nle Cys Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Cys

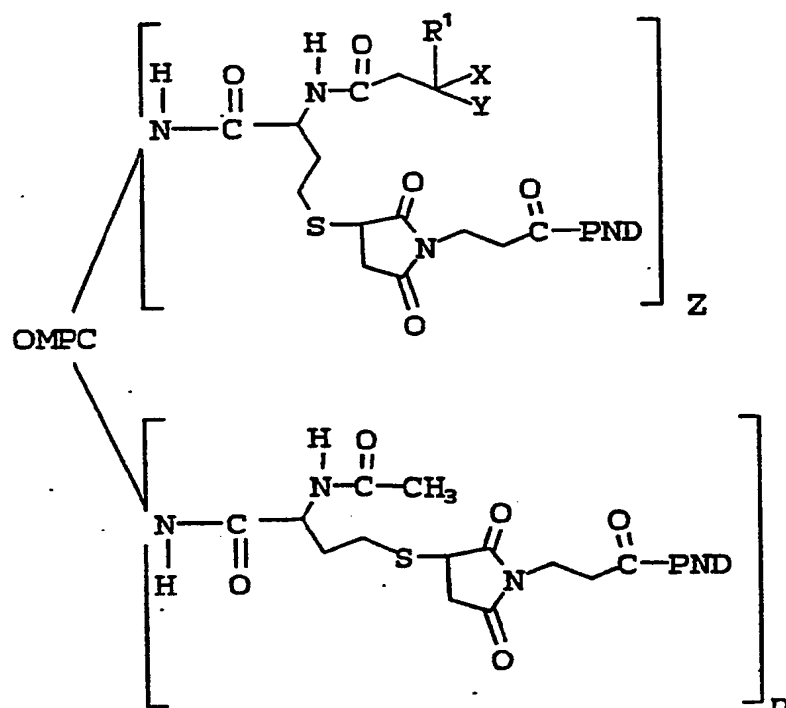
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Each of these peptides is preferably presented as a cyclic peptide, either through disulfide bonding (e.g. cPND402, cPND078, cPND495, which may be prepared according to Example 32) or through amide bond formation wherein the alpha or epsilon amino group of lysine on one side of the -Gly Pro Gly Arg - tetramer is bonded to the carboxy terminus of the

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peptide (cPND815, cPND955, cPND502, cPND535, cPND718, or cPND724, which may be prepared according to Examples 13, 25 and 26). The other, free amino group of the lysine is then used either to link the peptide to a marker amino acid such as Nle which itself is linked to the carrier protein through the anionic thiolactone spacer, or the Lys can be directly linked to the carrier through an anionic lactone spacer without the intervening marker amino acid.

In another preferred embodiment of the invention, the conjugate immunogen has the formula:



wherein "n" is that fraction of OMPC amines which cannot be saturated by addition of more anionic spacers, but which is available to addition by neutral spacers. "n" is preferably  $1 \leq 3000$ .

The conjugate immunogen of this invention is prepared by a process which comprises:

- a) Thiolyating the OMPC of Neisseria meningitidis b with an anionic thiolactone;
- 5 b) Suspending the thiolated OMPC of step a) in a buffer containing about 0.5 % deoxycholate;
- c) Maleimidating or bromoacetylating an HIV PND peptide;
- d) Reacting the maleimidated or bromoacetylated peptide of step c) with the thiolated OMPC of step 10 b); and
- e) Isolating the conjugate from the reaction of step d). This can most easily be accomplished by ultracentrifugation of the conjugation reaction and resuspension of the conjugate in a pharmaceutically 15 acceptable medium.

The conjugate immunogen of this invention is useful to induce immune responses in a mammal against the peptidyl epitope. Immunologically effective 20 amounts of the immunogen should be administered intramuscularly, subcutaneously, intravenously, intraperitoneally, or by any other route found to be efficacious in achieving exposure of the immunogen to the immune responsive system.

By intramuscular administration, a dose of 25 immunogen of about 0.1 µg to 1 mg per kilogram, and preferably about 10 µg - 500 µg per kilogram of immunogen is administered, in a pharmaceutically composition including an inert carrier. An adjuvant such as Freund's complete or incomplete, or the Ribi 30 adjuvant may be admixed with the conjugate immunogen prior to administration. It is also beneficial to



adsorb the immunogen to aluminum hydroxide prior to administration. Furthermore, antivirals, immunomodulators, antibacterials or other pharmaceuticals formulated as compositions comprising the conjugates of this invention may be co-administered to advantage.

In a preferred method of using the conjugate of this invention, an HIV principal neutralizing determinant is administered as a conjugate with the OMPC of Neisseria meningitidis b. The conjugate is administered intramuscularly at a dose of about 10 µg/kilogram, adsorbed to about 500 µg/ml of aluminum hydroxide at a concentration of 300 µg/ml conjugate, or a total dose of about 300 µg of the adsorbed conjugate is administered.

The following examples are provided to further illustrate the invention but should not be construed as limiting invention. In the examples that follow, all manipulations of thiolated OMPC were carried out in a nitrogen purged dry-box. The nitrogen was sterilized by passage through a 0.2 µM (Gelman Acro 50) filter. An effort was made to keep reagents and equipment sterile. All centrifugations unless otherwise noted were performed in a Beckman L-80 Optima centrifuge using a 80 Ti rotor and 10 mL polycarbonate tubes. All centrifugations were effected at 43,000 rpm, for 2 h at 4°C unless

otherwise noted. Amino acid analyses were done on a Beckman 6300 amino acid analyzer . NMR spectra were obtained on Varian 300 MHz and 400 MHz instruments. The protein titers were determined by the method of Lowry [Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275] using a Zymate II robotic system; thiol titers were determined by the method of Ellman [Ellman, G.L. (1959) , Arch. Biochem. Biophys. 82 , 70-77]; pKa values were determined by titration. Numbers referring to compounds refer to the foregoing figures. Note that the anionic thiolactones are enantiomers at the 2-position and are used as racemic materials.

#### EXAMPLE 1

**D,L-N-Succinylhomocysteine thiolactone, NSUTL.**  
D,L-Homocysteine thiolactone hydrochloride, (1.84 g, 12 mmol) was suspended in 20 mL of DMF and to it was added 1.04 g (10 mmol) of succinic anhydride and 1.79 mL of N,N-diisopropylethylamine (DIEA) (10 mmol).. The mixture was stirred at room temperature for 1 h after which an additional 0.9 mL of DIEA (0.5 mmol) was added. Stirring was continued for another 45 min after which a third charge of DIEA (0.9 mL, 5 mmol) was added. After stirring for an additional 2 h at room temperature the mixture was stored at 4°C overnight. It was then quenched onto 22 g of ice (pH=7.1) and the solution applied to a 30 mL column of Dowex 1 (acetate form). This was washed free of thiolactone and then eluted with 2M formic acid. The uv positive fractions were concentrated to 2.13 g of

N-succinylhomocysteine thiolactone, mp 128-135°C. FAB MS showed the major peak at m/z 218 (MH<sup>+</sup>). A small amount (47 mg) of this material was recrystallized by dissolving in acetonitrile, filtering and precipitating crystals with equal volumes of chloroform and cyclohexane affording pure product, mp 140-142°C. <sup>1</sup>HNMR (CDCl<sub>3</sub>) δ 4.67 (dd, 1H, J=7.0, 12.7), 3.44 (dt, 1H, J=5.4, 11.5), 3.33 (ddd, 1H, J=1.2, 7.1, 11.5), 2.55-2.7 (m, 5H), 2.20 (dq, 1H, J=7.1, 12.2). Anal. Calcd for C<sub>8</sub>H<sub>11</sub>NO<sub>4</sub>S: C, 44.24; H, 5.07; N, 6.45; S, 14.75. Found: C, 44.17; H, 5.04; N, 6.43; S, 14.48.

#### EXAMPLE 2

N-(3-Carboxymethylglutaryl)-homocysteine thiolactone, CMGTL. A mixture of carboxymethylglutaric anhydride, (1.72 g, 10 mmol) and homocysteine thiolactone hydrochloride, (1.85 g, 12 mmol) in dry DMF (10 mL) was stirred in a flask capped with a septum, cooled in an ice bath and DIEA (4.3 mL, 25 mmol) was added via syringe through the septum over a period of 15 min. After ageing the mixture for 2 hr, a stream of nitrogen was passed through the solution for 15 min to remove excess amine and then 15 g of ice was added. The pH at this point was 7. The solution was applied to a column of Dowex 1, (acetate form, 80 mL) which was eluted with water (250 mL) followed by 2N formic acid (350 mL). The acidic fraction was concentrated to 20 mL and applied to a column of neutral polystyrene resin (80 mL of HP-20, Pharmacia Fine Chemicals) and washed with 150 mL water. The product was recovered by elution with 250 mL of 12%

acetonitrile -water and lyophilized . This afforded 2.2 g of a material which was crystallized from acetonitrile-water affording 1.9 g of CMGTL. mp. 149-150 °C. MS ( FAB ) m/z 290 [MH<sup>+</sup>]. <sup>1</sup>HNMR (D<sub>2</sub>O) δ 4.68 (dd, 1H, J= 7.0,12.8), 3.45 (dt, 1H, J=5.5,11.4), 3.35 (ddd, 1H J=1.3,7.0,11.4), 2.71 ( m, 1H, J=7.0), 2.62 (m, 1H) 2.52 (dABq, 2H), 2.50 (d, 2H, J=7.0), 2.43 (dABq, 2H), 2.22 (dq, 1H, J=7.0,12.8). Anal. Calcd for C<sub>11</sub>H<sub>15</sub>NO<sub>6</sub>: C, 45.67; H, 5.23; N, 4.84; S, 11.08. Found: C, 45.64; H, 5.34, N, 5.05; S, 11.28.

### EXAMPLE 3

**D,L-N-(3-Bromopropionyl) homocysteine thiolactone.** D,L Homocysteine thiolactone hydrochloride, (52.0 g, 0.34 mol) was dissolved in 135 mL of water and covered with 300 mL of ethyl acetate. While stirring at 0 °C, 39 mL of 3-bromopropionyl chloride (0.78 mol) was added in 2 mL aliquots alternately with 20 mL aliquots of 2.5 N sodium hydroxide maintaining the pH at 7.35. After ageing at room temperature for 1 h without stirring, a yellow precipitate (76 g) was filtered. This precipitate was dissolved in 600 mL of refluxing ethyl acetate and filtered, hot, through Celite. After 72 h at 4 °C the title compound (40.5 g, 0.16 mol) was obtained. The supernatant fluid was reduced to 200 mL and seeded. After 18 h at 4 °C an additional 9.6 g was obtained. A total of 50.1 g of the title compound was isolated (58% yield): mp 142.5-149.1 °C. <sup>1</sup>HNMR (CDCl<sub>3</sub>) δ 6.06 (s, 1H), 4.51 (pentet, 1H, J=8. ), 3.6 (m, 2H), 3.36 (dt, 1H, J=6.8,15), 3.26 (dd, 1H, J=8.8,15), 2.98 (m, 1H), 2.81 (m, 2H), 1.94 (dq, 1H, J=9.2,16.6). Anal. Calcd for C<sub>7</sub>H<sub>10</sub>NO<sub>2</sub>SBr: C, 33.34; H, 4.01; N, 5.56; S, 12.72; Br, 31.69. Found: C, 33.60; H, 3.88; N, 5.51; S, 12.86; Br, 31.78.

EXAMPLE 4

D,L-N-(3-Dimethylphosphonopropionyl)homocysteine thiolactone. D,L -N-(3-Bromopropionyl)homocysteine thiolactone, (5 g, 19.8 mmol) was covered with 60 mL of trimethylphosphite. This solution, with nitrogen bubbling through it, was refluxed at 119 °C for 7 h. The excess trimethylphosphite was removed by distillation at 48 °C under vacuum leaving a clear yellow oil. Ethyl ether was slowly added to the oil with vigorous stirring to produce a white precipitate (5.7 g). The precipitate was recrystallized by dissolving it in 200 mL of boiling toluene, and adding hexane to the cloud point. White crystals, of the title compound, (3.4 g, 61% ) were filtered after ageing 72 h at 4 °C; mp 109.5-111.5 °C; <sup>1</sup>HNMR (CDCl<sub>3</sub>) δ 6.5 (s,1H), 4.50 (pentet,1H, J=6.5), 3.72 (2d, 6H), 3.33 (dt,1H, J=5.1,11.5), 3.24 (ddd,1H, J=1.3,7.0,11.5), 1.86 (m,1H), 2.53 (m,2H), 2.1 (dt,2H, J=7.75,17.86), 1.95 (dq,1H, J=7.1,12.4). Anal. Calcd for C<sub>9</sub>H<sub>16</sub>NPO<sub>5</sub>S: C, 38.43; H, 5.74; N, 4.98; P, 11.01; S,11.40. Found: C, 38.31; H, 5.59; N, 4.90; P, 11.24; S, 11.30.

EXAMPLE 5

D,L-N-(3-Phosphonopropionyl)homocysteine thiolactone, NPHTL:

D,L-N-(3-Dimethylphosphonopropionyl) homocysteine thiolactone, (1.0 g, 3.6 mmol) was dissolved in 18 mL of dichloromethane, bromotrimethylsilane (7.5 mL, 56.8 mmol) added and the reaction aged at 25 °C for 16 h. The solvent and volatiles were removed with a stream of N<sub>2</sub>, 100 mL of methanol added, and the

solution aged at 25 °C for 2 h. The methanol was then removed in vacuo. The remaining orange oil was dissolved in 8 mL of water, charged to 7 mL of Dowex 1X8 ( 200-400 mesh, acetate form ), and the column then washed with 250 mL of water. The NPHTL was eluted with 200 mL of 2 N formic acid. Complete removal of formic acid under reduced pressure gave a clear oil (0.78 g, 3.1 mmol, 87 % yield) which crystallized after 24 h at 25 °C. <sup>1</sup>HNMR (D<sub>2</sub>O) δ 4.71 (dd, 1H, J=7.1, 12.6), 3.47 (dt, 1H, J=5.4, 11.5), 3.36 (dd, 1H, J=7.1, 11.5), 2.54-2.68 (m, 3H), 2.23 (dq, 1H, J=7.1, 12.6), 2.06 (dt, 2H, J=8.7, 17.4). MS (FAB) m/z 252 (MH<sup>+</sup>). Anal. Calcd for C<sub>7</sub>H<sub>12</sub>NP<sub>5</sub>S · 0.25H<sub>2</sub>O: C, 32.62; H, 4.89; N, 5.44; P, 12.02; S, 12.44. Found: C, 32.59; H, 4.81; N, 5.43; P, 12.03; S, 12.33.

#### EXAMPLE 6

**N-(3-Sulfopropionyl)homocysteine thiolactone sodium salt, NSFTL.** A solution of N-(3-Bromopropionyl) homocysteine thiolactone, ( 1.0 g , 3.9 mmol) in DMF (6 mL) was added dropwise to a solution of sodium bisulfite (1.25 g, 12 mmol) in 16 mL of a 3:1 H<sub>2</sub>O:DMF mixture. The pH was adjusted to 8.8 by the addition of DIEA (1.5 mL), and the milky suspension stirred at room temperature for 18 hr. The mixture was filtered to remove undissolved sodium sulfite and the filtrate evaporated in vacuo to a solid. This was redissolved in 10 mL of water and charged to a 60 mL column of Dowex 50X4 (H<sup>+</sup>form) and eluted with 125 mL of water. The eluate was evaporated to 5 mL, adjusted to pH 7.2 with 5 N NaOH and applied to a column of brominated polystyrene resin (90 mL, SP-207, Mitsubishi Chem.

Co.). The column was eluted with water and after a forerun of 90 mL (AgNO<sub>3</sub> positive), 200 mL (uv positive) was collected and evaporated to dryness. The solid was dissolved in 15 mL of water and the product precipitated by the slow addition of 15 mL of absolute ethanol yielding 0.6 g (54%) of an amorphous white solid. <sup>1</sup>HNMR (D<sub>2</sub>O) δ 4.70 (dd, 1H, J=7, 12.5), 3.46 (dt, 2H, J=5.5, 11.7), 3.36 (ddd, 1H, J=1.4, 7.0, 11.7), 3.19 (m, 2H), 2.74 (m, 2H), 2.64 (dddd, 1H, J=1.4, 5.5, 7.0, 12.5), 2.22 (dq, 1H, J=7, 12.5). Anal. (dried 80 °C/4 hr) Calcd for C<sub>7</sub>H<sub>10</sub>NO<sub>5</sub>S<sub>2</sub>.Na: C, 30.54; H, 3.66; N, 5.09; S, 23.29. Found: C, 30.28; H, 3.54; N, 4.98; S, 23.54.

#### EXAMPLE 7

N-(3-carboxymethyl-3-methyl-glutaryl)-Homocysteine Thiolactone. MCMGTL:

Following the procedure in Example 2, Homocysteine Thiolactone Hydrochloride, 1.85 g (12 mmol) is acylated with 3-carboxymethyl-3-methyl-glutaric anhydride, 1.86 g (10 mmol) in the presence of diisopropylethylamine, 4.3 ml (25 mmol) in 10 ml of dry DMF. The product, MCMGTL, is purified by chromatography on Dowex 1 (acetate form) resin followed by chromatography on neutral polystyrene resin (HP-20) and is isolated by evaporation of the appropriate eluate.

EXAMPLE 8N-Tricarballyl-homocysteine thiolactone. NTCTL

Following the procedure in example 2, the reaction of  
homocysteine thiolactone (184 mg) with  
tricarballylic anhydride (158 mg) and  
diisopropylethylamine (522 mL) gave  
N-tricarballyl-homocysteine thiolactone as an  
amorphous mixture of isomers.

EXAMPLE 9

N- $\alpha$ -Bromoacetyl-L-arginyl-L-arginyl-L-arginine. Resin  
bound triarginine which was tris -guanidino  
protected by 4-methoxy-2,3,6-trimethylbenzenesulfonyl  
(Mtr) groups was synthesized on a 0.180 mmole scale  
on a Milligen Model 9050 peptide synthesizer.  
Preloaded Fmoc-Arg(Mtr)-OPKA resin (Milligen Co.)  
was coupled with Fmoc-Arg(Mtr) pentafluorophenyl  
esters using single 60 min reactions and a  
piperidine deprotection. The final resin was rinsed  
with methylene chloride and ether and dried in vacuo.  
A Kaiser test was positive for amine. This resin  
with the bound tripeptide derivative was swollen in 7  
mL of N-methylpyrrolidine (NMP) and then 31  $\mu$ L of  
DIEA and 85 mg of p-nitrophenyl bromoacetate  
added. The resin was agitated at RT until a negative  
Kaiser test was observed. The bromoacetylated  
peptidyl resin was then washed with DMF (50 mL),  
methylene chloride (50 mL) and ether (50 mL).  
Cleavage from the resin was effected by TFA (20 mL)  
containing 3% thioanisole for 7 h at RT. The resin  
was removed by filtration, washed with TFA (3 x 20



mL) and the combined filtrates concentrated in vacuo. The residue was triturated with ether affording crude product (82 mg) which was purified by HPLC (Waters RCM 25 x 10 Delta Pack C<sub>18</sub>, 10 mL/min, using a gradient [8% to 15 % acetonitrile (0.1% TFA) in 20 min] affording 14.6 mg of Na-bromoacetyl-L-arginyl-L-arginyl-L-arginine. FAB MS m/z 607 (MH<sup>+</sup>).

#### EXAMPLE 10

Thiolation of OMPC with N-Succinylhomocysteine thiolactone and reaction with Bromoacetyltriarginine

5 ml of OMPC (6 mg/ml) was ultracentrifuged at 43,000 rpm, 4°C for two hours. The OMPC pellet was resuspended using a DOUNCE homogenizer, in 8 ml of thiolyating solution ( 85 mg EDTA, 17 mg DTT, and 120 mg of N-succinyl-homocysteine thiolactone in 10 ml of 0.1M, pH 11 borate buffer). The thiolation was allowed to proceed at room temperature for 18 hours under N<sub>2</sub>, and the solution was then centrifuged at 43,000 rpm, 4°C for two hours. The pellet was resuspended in 10 ml of 0.1M pH 8 phosphate buffer, recentrifuged, and resuspended in 5 ml of pH 8 buffer. An Ellmans assay indicated a sulfhydryl titer of 0.64 mmoles/mL. To 4 ml of this suspension was added bromoacetyl triarginine (9 mg) and the mixture was aged at room temperature overnight. The mixture was centrifuged at 3000 rpm for two minutes and the supernatant sampled for analysis.

The protein content (Lowry) was 1.1 mg/mL.

EXAMPLE 11

Thiolation of OMPC with  
Phosphonopropionyl-homocysteine thiolactone and  
reaction with bromoacetyl triarginine.

5 Following the procedure in example 10, OMPC( 5 mL,  
3.8 mg/mL) was thiolated with phosphonopropionyl-  
homocysteine thiolactone , and the resulting  
thiolated OMPC (SH titer, 334 nM/mL) reacted with  
10 bromoacetyltriarginine providing a conjugate having  
1.38 mg/mL protein and a SCMHC content of 63 nM/mL.

EXAMPLE 12

Thiolation of OMPC with Carboxymethylglutaryl-  
homocysteine thiolactone and reaction with  
15 Bromoacetyltriarginine

OMPC was thiolated with CMGTL as in Example 10 and  
was found to have an Ellmans titer of 275 nM SH/ml.  
1 ml of this suspension was added to a vial  
containing 1.5 mg of bromoacetyl triarginine. The  
20 mixture was kept in the refrigerator overnight then  
centrifuged for 3 minutes at 3000 rpm and the  
supernatant sent for Lowry and aminoacid analyses.  
Lowry = 3.1 mg/ml; S-Carboxymethyl homocysteine = 193  
nM/ml. When Bromoacetyl triarginine was added to  
25 OPMC which was thiolated with the neutral reagent,  
N-Acetyl Homocysteine Thiolactone (Ellmans assay =  
399 nM SH/ml) and the reaction worked up as above,  
the Lowry assay was only 0.9 mg/ml.

EXAMPLE 13

Preparation of cPND724 (Seq. ID: 9:):

Nle-NH<sub>2</sub>

(e) |

Lys Acap Gln Arg Gly Pro Gly Arg Ala Phe Acap

The linear peptide (Seq. Id:9:)

Nle-NH-Cbz

|

Lys Acap Gln Arg Gly Pro Gly Arg Ala Phe Acap

was synthesized using Fmoc-Lys(e Nle)-OH, Fmoc chemistry and single coupling on an ABI-431 solid phase synthesizer, at 0.25 mmolar scale (Acap stands for 6-aminocaproic acid). The peptide was cleaved from the resin with trifluoroacetic acid (TFA) and anisole, 20°C, 4.5 hours. About 0.4 g. of crude material was purified on a Waters Delta Prep system, and the peak fraction was dried. Mass spectrometry analysis gave a molecular weight of 1473, which is consistent with the calculated molecular mass.

The linear peptide (0.34g, 0.22 mmol) was dissolved in 200 mL DMF, along with diisopropylethylamine (DIEA) (0.12 mL, 0.66 mmol), BOP (111 mg, 0.25 mmol), and HOBt (38 mg, 0.25 mmol). An additional aliquot of BOP (100 mg, 0.25 mmol) was added after analysis of an aliquot revealed the presence of residual linear peptide. After reacting for 18 hours at room temperature, a 1 mL aliquot was evaporated, redissolved in 50% acetic acid, and analyzed by HPLC. All of the linear

material was converted to cyclic peptide according to this analysis. Therefore, the entire reaction was dried, redissolved in 50% acetic acid, and purified by HPLC. The sample was loaded on a C<sub>18</sub> column equilibrated with aq. 0.1% TFA, and the peptide  
5 eluted over 60 minutes by gradient up to 80% CH<sub>3</sub>CN/0.1% TFA.

Peak HPLC fractions were combined, dried, redissolved in aq. alcohol, and hydrogenated over a  
10 palladium catalyst. The catalyst was filtered off and the sample was dried, and then dissolved in 50% acetic acid. The sample was repurified by HPLC, and peak fractions eluting over 70 minutes in an aq. 0.1% TFA - 70% CH<sub>3</sub>CN/0.1% TFA gradient were pooled and  
15 dried. Mass spectroscopic analysis gave a molecular weight of 1336, which is consistent with the calculated mass for cPND724.

#### EXAMPLES 14-15

20 Preparation of cPND955 (Seq. Id:3:), and cPND815  
(Seq. Id:2:)

Using essentially the same procedure as in  
25 Example 13, but varying the primary amino acid sequence the following compounds were prepared, and the molecular weight confirmed by mass spectroscopic analysis.

30

Peptide	Mass	EX #	Seq. Id
cPND955	1302	15	3

5 Nle-NH2  
e |  
Lys-Acap-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Acap

10

cPND815	929	16	2
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15

Nle-NH2  
e |  
Lys-His-Ile-Gly-Pro-Gly-Arg-Ala

EXAMPLE 16

Preparation of maleimido propionyl cPND955 (Seq. Id:3:)

20

To a cooled solution of cPND955 trifluoroacetate salt, (9.5 mg, in 0.8 ml of 70% acetonitrile) was added 61 ml. of 0.5 M sodium bicarbonate solution followed by 2.3 mg. of maleimidopropionyl (N-Hydroxy) succinimide. The solution was stirred in ice for 1 hr. and the course of the reaction was followed by reverse phase HPLC (30% MeCN/0.1% TFA). The reaction was quenched with 2.3 ml of TFA and the peptide was isolated by preparative HPLC using gradient elution from 20 to 40% MeCN in 0.1% TFA over 30 minutes. The peak eluting between 21.6-30.6 minutes was collected, concentrated and lyophilized. Wt 9.3 mg.; FAB-MS, m/z 1454, M+H.

25

30

EXAMPLE 17Preparation of Maleimido Propionyl cPND815. MPP-cPND815(Seq. Id:2:)

To a solution of cPND815 (12 mg) in 1 ml of 2:1 MeCN/H<sub>2</sub>O was added sodium bicarbonate, (260 ul of 0.2M) and Maleimido Propionyloxy Succinimide (5 mg). The mixture was stirred in ice for 50 min then quenched with 4 µl. of TFA. The maleimido peptide was isolated by preparative HPLC (isocratic with 24% MeCN in 0.1% TFA for 10 minutes, then with a gradient of 24-34% for 10 minutes). The peak eluting between 16 to 24 minutes was concentrated and lyophilized providing MPP-cPND815 as a white powder. Wt. 10 mg; FAB-MS, m/z 1081, M+H.

EXAMPLE 18Thiolation of OMPC with CMGTL and reaction with Maleimido Propionyl Peptide-cPND815. (Seq. Id:2:)

OMPC (9 ml of 3.2 mg/ml) was ultracentrifuged at 43K, for 2 hrs. and the resulting pellet was resuspended in 8 mL of a solution of 78 mg CMGTL, 85 mg of EDTA and 18 mg of DTT in 10 ml of pH 11, 0.01 M Borate buffer. The suspension was flushed with nitrogen and kept at room temperature for two days, then ultracentrifuged and the pellet resuspended in 10 mL of 0.1 M pH7 phosphate buffer. This was ultracentrifuged and the pellet finally suspended in 7.6 ml of 0.1M pH 8 phosphate buffer. (Ellmans=0.387 µM SH/ml.) To 5.5 mL of this suspension was added 625 µL of a solution containing 3.39 µM/ml of Maleimido Propionyl cPND815 (ca. 10 mg

in 1.5 ml water). After 15 min an Ellmans titer for residual SH groups was negative. The suspension was dialyzed vs 4 L. of 0.01M pH8 phosphate overnight. (Lowry protein=2.16 mg/ml; Spinco NLe=175 nM/ml corresponding to a peptide loading of 7.5%). This conjugate was aqueous soluble. By contrast, the reaction of Maleimido Propionyl CPND815 with OMPC thiolated with the neutral reagent N-Acetylhomocysteine thioactone resulted in almost complete precipitation. The supernatant from this reaction gave a Lowry protein assay of 0.12 mg/ml.

#### EXAMPLE 19

Conjugation of CMGTL-OMPC with MPP-cPND955 (Seq. Id:3:):

OMPC was thiolated with CMGTL as in Example 18 and was found to have an Ellmans titer of 0.361  $\mu$ M SH/mL. To 5 mL of this suspension was added 0.65 mL of a solution (3.1  $\mu$ M/ml) of MPP-cPND955. The reaction mixture was dialyzed vs 4 L of 0.01M, pH7 phosphate buffer overnight at 4°C and then removed to a graduated tube: Vol. 5.8 ml, Lowry protein=2.84 mg/ml, Amino Acid Analysis Nle=219 nM/ml, Peptide loading = 10%. This conjugate was nicely aqueous soluble.

#### EXAMPLE 20

Thiolation of OMPC with MCMGTL and reaction with MPP-cPND955. (Seq. Id:3:):

Following the procedure of Example 18, OMPC is thiolated with N-(Carboxymethyl-3-methylglutaryl)-Homocysteine thiolactone and then reacted with Maleimido Propionyl cPND 955 affording an OMPC-MPP cPND 955 conjugate containing a 3-carboxymethyl-3 methylglutary substituted spacer.

#### EXAMPLE 21

Thiolation of OMPC with CMGTL and N-Acetyl-Homocysteine Thiolactone and conjugation to MPP-cPND955. (Seq. Id:3:):

OMPC (5ml of 4.3 mg/ml) was ultracentrifuged at 43K for 2 hr and the pellet was resuspended in 5 ml of a solution of 86 mg CMGTL, 85 mg of EDTA, and 17 mg of DTT in 10 ml of pH 11, 0.1 M borate buffer. The suspension was flushed with nitrogen and kept overnight at room temperature. Sodium Hydroxide, (100 µl of 5 N) and N-Acetyl Homocysteine Thiolactone Hydrochloride, (40 mg), were added and the mixture was tumbled overnight. The suspension was diluted with 0.1 M, pH 7 phosphate buffer (4 ml), ultracentrifuged, and the pellet resuspended in 7 ml of pH 7 buffer. The suspension was again ultracentrifuged and the pellet resuspended in 4.2 ml of pH 8, 0.1 M TED buffer, (Ellmans = 0.75 µM SH/ml). To 3 ml of this suspension was added MPP-cPND955, (1.2 ml of 2 µM/ml). After 1 hr. the suspension was dialyzed overnight at 4°C vs 4 l of pH 8. 0.01 M phosphate buffer. The final dialysate volume was 4.6 ml; Lowry protein = 1.94 mg/ml, Spinco Nle = 387 nM/ml, Peptide loading = 29%.



EXAMPLE 22

Conjugation of CMGTL-OMPC with MPP-cPND955 (Seq. Id:3:) and MPP-cPND815 (Seq. Id:2:):

5 Following the procedure of examples 18, OMPC is thiolated with CMGTL and then reacted with about an equimolar amount of MPP-cPND955 and MPP-cPND815 to form a conjugate having different peptidyl epitopes.

EXAMPLE 23

10 Thiolation of OMPC with NSUTL, MCMGTL, NTCTL, or NSHTL, and conjugation with MPP-cPND955 (Seq. Id:3:), MPP-cPND815 (Seq. Id:2:), or MPP-cPND955 and MPP-cPND815:

15 Using NSUTL, NTCTL, MCMGTL, or NSHTL, OMPC is thiolated following the procedure of example 19 and is then reacted with with MPP-cPND955, MPP-cPND815, or MPP-cPND955 and MPP-cPND815, according to examples 18, 19 or 22 respectively.

EXAMPLE 24

20 Solution Synthesis of Peptide Bonded cPND7:

The linear peptide Cbz-Nle-Lys(Boc)-His(Trt)-Ile-Gly-Pro-Gly-Arg(Mtr)-Ala-Phe (SEQ. ID:8) was synthesized following solid-phase methods on an ABI 431A peptide synthesizer using 373 milligrams (0.1  
25 mmoles) of commercially available Fmoc-Phenylalanyl-p-alkoxy-benzyl alcohol resin. With the exception of norleucine, which was purchased in the benzyloxy-carbonyl (Cbz, or Z-Nle) protected form, L-amino acids used were the  
30 fluorenylmethoxycarbonyl (Fmoc) derivatives having the appropriate acid-labile side chain protecting

groups. The polypeptide-derivatized resin product was transferred to a sintered glass funnel, washed with dichloromethane, and dried, to yield 0.6 g of polypeptide-resin product.

5 The peptide was cleaved from the resin by treatment with 6 ml of a 95:2:3 mixture of TFA:1,2 ethanediol:anisole for 16 hours. The reaction mixture was filtered through a sintered glass funnel, the resin washed with 10 ml TFA, and the filtrates combined. Following concentration to about 1 to 2 ml of yellow oil, the linear peptide was recovered by trituration with 400 ml of diethyl ether, in 50 ml portions, and filtration on a sintered glass funnel. Dissolution with 100 ml 1% TFA followed by lyophilization yielded 298 mg of linear peptide.

15 The peptide powder was dissolved in 800 ml DMF, neutralized with 0.42 ml diisopropylethylamine, and treated with 0.077 ml diphenylphosphorylazide. The solution was stirred in the dark for 70 hours at 4°C to allow formation of the cyclic lactam. After quenching by addition of 3 ml glacial acetic acid, the reaction mixture was concentrated to about 1 to 2 ml of oil, dissolved in 10% aqueous acetic acid, and lyophilized.

25 The cyclic peptide was purified by G-15 size exclusion chromatography using 5% acetic acid as the mobile phase. Fractions, monitored by UV detection, containing the peptide were pooled and lyophilized to yield 135 mg of dry cyclic peptide. All results obtained were consistent with a cyclic peptide wherein the cycle is formed through the (ε) amino group of lysine and the carboxy terminal phenylalanine carboxyl group, having the structure cPND7: (Seq. Id:8:)



Deprotection of cPND7 to yield the hydrogen form, cPND8:

Deprotection of cPND7 was achieved by dissolving the cyclic peptide in 20 ml of 30% aqueous acetic acid and hydrogenation at 40 psi for 16 hours over 100 mg of 10% palladium on carbon. The reaction mixture was filtered over celite to remove the catalyst, and the filtrate was lyophilized. Reverse phase HPLC using a Vydac C18 semi-prep column was utilized to obtain 8.5 mg of pure deprotected cyclic peptide. This method of deprotection is applicable to all peptides synthesized as the benzyloxycarbonyl N-protected peptide, to yield the free hydrogen form of the peptide. The structure of the product was confirmed by FAB-MS, analytical HPLC and amino acid analysis, and all results were consistent with the structure cPND8 (Seq. Id:8:)



EXAMPLE 26Preparation of *Neisseria meningitidis* B11 Serotype 2 OMPC**A. Fermentation****1. *Neisseria meningitidis* Group B11**

A tube containing the lyophilized culture of *Neisseria meningitidis* (obtained from Dr. M. Artenstein, Walter Reed Army Institute of Research (WRAIR), Washington, D.C.) was opened and Eugonbroth (BBL) was added. The culture was streaked onto Mueller Hinton agar slants and incubated at 37°C with 5% CO<sub>2</sub> for 36 hours, at which time the growth was harvested into 10% skim milk medium (Difco), and aliquots were frozen at -70°C. The identity of the organism was confirmed by agglutination with specific antiserum supplied by WRAIR, and typing serum supplied by Difco.

A vial of the culture from the second passage was thawed and streaked onto 10 Columbia Sheep Blood agar plates (CBAB-BBL). The plates were incubated at 37°C with 5% CO<sub>2</sub> for 18 hours after which time the growth was harvested into 100 mL of 10% skim milk medium, aliquots were taken in 0.5 mL amounts and frozen at -70°C. The organism was positively identified by agglutination with specific antiserum, sugar fermentation and gram stain.

A vial of the culture from this passage was thawed, diluted with Mueller-Hinton Broth and streaked onto 40 Mueller-Hinton agar plates. The plates were incubated at 37°C with 6% CO<sub>2</sub> for 18

hours after which time the growth harvested into 17 mL of 10% skim milk medium, aliquotted in 0.3 mL amounts and frozen at -70°C. The organism was positively identified by Gram stain, agglutination with specific antiserum and oxidase test.

## 2. Fermentation and collection of cell paste

a. Inoculum Development- The inoculum was grown from one frozen vial of Neisseria meningitidis Group B, B-11 from above (passage 4). Ten Mueller-Hinton agar slants were inoculated, and six were harvested approximately 18 hours later, and used as an inoculum for 3 250 mL flasks of Gotschlich's yeast dialysate medium at pH 6.35. The O.D.<sub>660</sub> was adjusted to 0.18 and incubated until the OD<sub>660</sub> was between 1 and 1.8. 1 mL of this culture was used to inoculate each of 5 2L. Erlenmeyer flasks (each containing 1 liter of medium; see below) and incubated at 37°C in a shaker at 200 rpm. The O.D. was monitored at hourly intervals following inoculation. 4 liters of broth culture, at an O.D.<sub>660</sub> of 1.28 resulted.

70 Liter Seed Fermenter- Approximately 4 liters of seed culture was used to inoculate a sterile 70-liter fermenter containing about 40 liters of complete production medium (see below). The conditions for the 70-liter fermentation included 37°C, 185 rpm with 10 liters/minute air sparging and constant pH control at about pH 7.0 for about 2 hours. For this batch, the final O.D.<sub>660</sub> was 0.732 after 2 hours.

## 800-Liter Production Fermenter

Approximately 40 liters of seed culture were used to inoculate a sterile 800 liter fermenter containing 568.2 liters of complete production medium (see below). The batch was incubated at 37°C, 100 rpm with 60 liters/minute air sparging and constant pH control at pH 7.0. For this batch, the final O.D. was 5.58 thirteen hours after inoculation.

3. Complete Medium for Erlenmeyer flasks and 70-and 800-liter fermenters

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Fraction A

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L-glutamic acid	1.5 g/liter
NaCl	6.0 g/liter
Na <sub>2</sub> HPO <sub>4</sub> .anhydrous	2.5 g/liter
NH <sub>4</sub> Cl	1.25 g/liter
KCl	0.09 g/liter
L-cysteine HCl	0.02 g/liter

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Fraction B (Gotschlich's Yeast Dialysate):

1280 g of Difco Yeast Extract was dissolved in 6.4 liters of distilled water. The solution was dialyzed in 2 Amicon DC-30 hollow fiber dialysis units with three H10SM cartridges. 384 g MgSO<sub>4</sub>.7-H<sub>2</sub>O and 3200 g dextrose were dissolved in the dialysate and the total volume brought to 15 liters with distilled water. The pH was adjusted to 7.4 with NaOH, sterilized by passage through a 0.22 µ filter, and transferred to the fermenter containing Fraction A.

For the Erlenmeyer flasks: 1 liter of Fraction A and 25 mL of Fraction B were added and the pH was adjusted to 7.0-7.2 with NaOH.

5 For the 70 liter fermenter: 41.8 liters of Fraction A and 900 mL of Fraction B were added and the pH was adjusted to 7.0-7.2 with NaOH.

For the 800 liter fermenter: 553 liters of Fraction A and 15.0 liters of Fraction B were added and the pH was adjusted to 7.1-7.2 with NaOH.

10 d. Harvest and Inactivation

After the fermentation was completed, phenol was added in a separate vessel, to which the cell broth was then transferred, yielding a final phenol concentration of about 0.5%. The material was held at room temperature with gentle stirring until the culture was no longer viable (about 24 hours).

e. Centrifugation

15 After about 24 hours at 4°C, the 614.4 liters of inactivated culture fluid was centrifuged through Sharples continuous flow centrifuges. The weight of the cell paste after phenol treatment was 3.875 kg.

B. OMPC Isolation

25 Step 1. Concentration and diafiltration

The phenol inactivated culture was concentrated to about 30 liters and diafiltered in sterile distilled water using 0.1 micro-hollow fiber filters (ENKA).

30

Step 2. Extraction

5 An equal volume of 2X TED buffer [0.1 M TRIS 0.01 M EDTA Buffer, pH 8.5, with 0.5% sodium deoxycholate] was added to the concentrated diafiltered cells. The suspension was transferred to a temperature regulated tank for OMPC extraction at 56°C with agitation for 30 minutes.

10 The extract was centrifuged at about 18,000 rpm in a Sharples continuous flow centrifuge at a flow rate of about 80 mL/minute, at about 4°C. The viscous supernatant was then collected and stored at 4°C. The extracted cell pellets were reextracted in TED buffer as described above. The supernatants were pooled and stored at 4°C.

Step 3. Concentration by Ultrafiltration

15 The pooled extract was transferred to a temperature regulated vessel attached to AG-Tech 0.1 micron polysulfone filters. The temperature of the extract was held at 25°C in the vessel throughout the concentration process. The sample was concentrated tenfold at an average transmembrane pressure of between 11 and 24 psi.

Step 4. Collection and Washing of the OMPC

25 The retentate from Step 3 was centrifuged at about 160,000 x g (35,000 rpm) at about 70°C in a continuous flow centrifuge at a flow rate between 300 to 500 mL/minute, and the supernatant was discarded.

30 The OMPC pellet was suspended in TED Buffer (190 mL buffer; 20 mL/g pellet) Step 2 and Step 4 were repeated twice (skipping Step 3).



Step 5. Recovery of OMPC Product

The washed pellets from Step 4 were suspended in 100 mL distilled water with a glass rod and a Dounce homogenizer to insure complete suspension. The aqueous OMPC suspension was then filter sterilized by passage through a 0.22  $\mu$  filter, and the TED buffer was replaced with water by diafiltration against sterile distilled water using a 0.1  $\mu$  hollow fiber filter.

EXAMPLE 27

N-a-Bromoacetyl-L-arginyl-L-arginyl-L-arginine (10). Resin bound triarginine which was tris-guanidino protected by 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) groups was synthesized on a 0.180 mmole scale on a Milligen Model 9050 peptide synthesizer. Preloaded Fmoc-Arg(Mtr)-OPKA resin (Milligen Co.) was coupled with Fmoc-Arg(Mtr) pentafluorophenyl esters using single 60 min reactions and a piperidine deprotection. The final resin was rinsed with methylene chloride and ether and dried in vacuo. A Kaiser test was positive for amine. This resin with the bound tripeptide derivative was swollen in 7 mL of N-methylpyrrolidine (NMP) and then 31  $\mu$ L of DIEA and 85 mg of p-nitrophenyl bromoacetate added. The resin was agitated at RT until a negative Kaiser test was observed. The bromoacetylated peptidyl resin was then washed with DMF (50 mL), methylene chloride (50 mL) and ether (50 mL). Cleavage from the resin was

effected by TFA (20 mL) containing 3% thioanisole for 7 h at RT. The resin was removed by filtration, washed with TFA (3 x 20 mL) and the combined filtrates concentrated in vacuo. The residue was trituated with ether affording crude product (82 mg) which was purified by HPLC (Waters RCM 25 x 10 Delta Pack C<sub>18</sub>, 10 mL/min, using a gradient [8% to 15 % acetonitrile (0.1% TFA) in 20 min] affording 14.6 mg of N<sub>α</sub>-bromoacetyl-L-arginyl-L-arginyl-L-arginine. FAB MS m/z 607 (MH<sup>+</sup>).

#### EXAMPLE 28

Preparation of Phosphono-Conjugate in the presence and absence of Detergent:

I. Phosphono-Conjugate Preparation in the Absence of Detergent:

OMPC (10 mL, 3.2 mg/mL) was pelleted and resuspended in 10 mL of thiolation mixture (EDTA, DTT, lactone in pH 11, 0.1 M Borate buffer). The sample was degassed and incubated at 34°C for 14 hours. Phosphonothiolactone (75 mg) was added and incubated at 34°C for an additional 8 hours. The thiolated OMPC was then centrifuged and resuspended in 0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 8, and repelleted. The pellet was resuspended in 0.01 M, pH 8 phosphate (5.5 mL). An Ellman assay gave a sulfhydryl titer of 103 nmol/mg. A solution containing 8 mg of maleimidated cPND495 in 1.0 mL of 0.1 M, pH 8 phosphate buffer was

added (final phosphate concentration was 0.024 M). The conjugation was allowed to proceed overnight. Upon completion, the sample was found to have precipitated.

5 2. Phosphono Conjugate formation in the presence of Detergent:

10 OMPC (10 mL, 3.2 mg/mL) was pelleted (2 hours, 43,000rpm, 4°C). The pellet was resuspended in 10 mL of sterile filtered thiolation mixture (10 mL pH 11, 0.1 M borate buffer, 85 mg EDTA, 15 mg DTT, 75 mg NPHTL). The pH was adjusted to 11.4 by addition of 5N NaOH. The solution was degassed and allowed to  
15 react overnight at room temperature. The reaction was centrifuged, and the pellet resuspended in 0.1 M, pH 8 phosphate buffer. The sample was repelleted and resuspended in TED buffer (8 mL). An Ellman assay gave a sufhydryl titer of 0.281  $\mu\text{mol SH/mL}$ , or 70  
20 nmol SH/mg OMPC. Maleimidated cPND495 (500  $\mu\text{L}$ , 1.335  $\mu\text{mol}$ ) was added to 4.75 mL of the thiolated OMPC, and the conjugation was allowed to proceed overnight. The sample had no precipitate. The conjugate was pelleted at 43,000 rpm, 4°C, 2 hours, and resuspended  
25 in pH8, 0.03 M phosphate buffer; and analyzed by Lowry and amino acid analysis:

Lowry: 1.6 mg/mL  
Nle : 58.0 nmol/mL  
30 b-Ala: 65.6 nmol/mL  
Lys : 614.8 nmol/mL  
loading: 41 nmol peptide/mg OMPC.

EXAMPLES 29-31

All of the following peptides were conjugated to OMPC which was thiolated with NPHTL essentially as described in Example 29. The thiolated OMPC was resuspended in TED buffer and the indicated peptide, which had previously been maleimidated, was reacted with the thiolated OMPC:

EXAMPLE - PEPTIDE SEQUENCE

EXAMPLE 29 - cPND502 SEQ ID:4

Lys Abu His Ile Ile Gly Pro Gly Arg Ala Phe

EXAMPLE 30 - cPND535 SEQ ID:5

Lys Abu Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala  
Phe Val Thr Ile Gly

EXAMPLE 31 - cPND078 SEQ ID:6

Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Cys

Results:

In every case, the conjugation proceeded in TED to yield a soluble conjugate. When rabbits were immunized with these conjugates the following results were achieved for development of neutralizing antibodies at 10 weeks after immunization (#) or at 12 weeks after immunization (\*):

Peptide in conjugate	Number of Rabbits Developing Neutralizing Antibodies
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cPND502	2/4#
cPND535	4/4#
cPND078	3/3*

### EXAMPLE 32

#### Synthesis of Disulfide Bonded cPND402:

1. SYNTHESIS OF: H-Nle Cys Tyr Asn Lys Arg Lys Arg  
Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys  
Asn Ile Ile Gly Cys-OH ( $C_{135}H_{220}N_{42}O_{33}S_2$ , formula  
weight = 3023.6). Seq. Id:7:

The 26mer was assembled on the Milligen #  
9050 synthesizer, starting from the partially  
racemised Fmoc-L-Cys(Trt)-OPKA resin (Milligen batch  
B 090426) using 0.081 meq/g (about 604 mg), using  
2.47 g (0.200 meq). The resin was mixed with an  
equal volume of glass beads (Sigma 150-212  $\mu$ m). The  
mixture completely filled two 1 x 10 ccm columns,  
connected in series. Reagents were Fmoc-Pft ester  
(except for threonine, which was dHBt), using in four  
fold molar excess in N-methyl pyrrolidine solvent.  
Side chain protection was: Y (tert-butyl); K (Boc); R  
(Mtr); His (Boc); T (tert-butyl); C (Trt). The  
protocol was modified to give double coupling with  
K<sup>7</sup>; I<sup>9</sup>; I<sup>11</sup>; G<sup>12</sup>; P<sup>13</sup>; G<sup>14</sup>; R<sup>15</sup>; F<sup>17</sup>; Y<sup>18</sup>; T<sup>19</sup>; T<sup>20</sup>;  
I<sup>23</sup>; I<sup>24</sup>. Acylation recycle times were extended from  
30 to 60 minutes for all units, except for G<sup>14</sup> and  
A<sup>16</sup>, and to 90 minutes for I<sup>9</sup> (2x); I<sup>11</sup> (2x); I<sup>23</sup>  
(2x) and I<sup>24</sup> (2x). The derivatized resin was  
maintained as the free terminal amine which was  
washed with CH<sub>2</sub>Cl<sub>2</sub> and air-dried.

The mixture of dry derivatized resin and glass beads was resuspended in 95% TFA, 4% ethane dithiol, 1% CH<sub>3</sub>SPh (30 mL) at 23°C in a sealed flask, with gentle stirring on an oscillating tray for 8 hours. The bright yellow mixture was then filtered and the insolubles were thoroughly extracted with 100% TFA (3 x 20 mL). The combined dark orange filtrates were evaporated to give a pale tan, oily gum. On trituration with ether (20 mL) this material instantly became a colorless solid, which was transferred to a filter by trituration with additional ether (3 x 20 mL). After drying, the crude product was obtained as a fine colorless powder (583 mg).

Analytical reverse phase HPLC on a 0.46 x 25.0 cm Vydac C<sub>18</sub> column of about a 50 µg sample, dissolved in aqueous 0.1% TFA/20% CH<sub>3</sub>CN, revealed a major component and a later eluting minor component. These were separately collected after injection of a 30 mg and another 50 mg aliquot of the sample onto two 2.21 x 25.0 cm preparative columns in series. A total of 35.2 mg of the earlier eluting material and 8.2 mg of the later eluting material was recovered following lyophilization. FAB-MS gave a [M+H]<sup>+</sup> = 3022.1 and an [M+Na] = 3044.2, which correlates with the calculated mass.

## 2. PREPARATION OF THE CYCLIC DISULFIDE:

H-Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Lys Asn  
Ile Ile Gly Cys-OH

(SEQ ID: 7:)

a. K<sub>3</sub>Fe(CN)<sub>6</sub> INDUCED OXIDATION:

The linear 26 mer dithiol compound (35.0 mg) was dissolved in degassed distilled water (38 mL) at 23°C to give a clear colorless solution at pH 2.73. The pH was adjusted to 8.5 with 0.1 N NH<sub>4</sub>OH, and the solution was covered with an atmosphere of nitrogen. An aliquot of the material was immediately run on analytical reverse phase HPLC and found to be undergoing oxidation as evidenced by the appearance of a early peak.

With magnetic stirring, a freshly prepared solution of 0.01 M K<sub>3</sub>Fe(CN)<sub>6</sub> was added by power driven hypodermic syringe at 23°C under nitrogen. Analysis of a small aliquot by HPLC revealed total conversion of starting material to an earlier elution time. The reaction mixture (pH 8.3) was mixed with 10% aqueous acetic acid and stirred to give a pH of 4.0. The solution was filtered to remove insoluble material, and the faintly yellow solution was evaporated and then lyophilized to give about 27.9 mg of a pale yellow powder. The material was dissolved in 0.1% TFA, 20% CH<sub>3</sub>CN and gradient eluted on a preparative HPLC. A major early eluting peak and a later eluting peak (4:1) were separately collected, lyophilized to yield 6.1 mg of the early and 1.5 mg of the late eluting materials. FAB-MS analysis of the early eluting material: [M+H]<sup>+</sup> 3019.7; [M+Na]<sup>+</sup> 3042.5; FAB-MS analysis of the late eluting material: [M+H]<sup>+</sup> 3021.0; [M+Na]<sup>+</sup> early material = 3041.5; all of which corresponds to the correct mass for the cyclized cPND402. The later eluting material is the D-cysteine isomer.

Amino acid analysis of the products gave the predicted amino acid compositions for the cyclized products and confirmed that the later eluting material is the D-cysteine containing diastereomer.

5

b. AIR OXIDATION:

The linear 26 mer prepared in (1) above (86 mg, 28.4  $\mu$ moles) was dissolved in aqueous 0.1% TFA, 20% acetonitrile (284 mL) at 23°C and the solution was allowed to stand open to the air.

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Cyclization was monitored by reverse phase HPLC and the sample was found to be almost completely converted to the early eluting material, with almost complete disappearance of starting linear material, by t = 24 hours. The clear, colorless solution was evaporated to about 8 mL at which point an additional 10 mg sample prepared in the same way as the 86 mg, was added. The combined sample was evaporated to about 9 mL. The cloudy colorless solution was subjected to HPLC separation, in two separate runs, on two 2.12 x 25.0 cm Vydac C<sub>18</sub> columns in series. Two peaks of material were separately collected, an early eluting peak and a later eluting peak. Each peak was separately evaporated and lyophilized to yield 30.1 mg and 9.7 mg of the early and late materials respectively. The early eluting material was combined with other preparations of early eluting cyclized material to yield a total of 47.5 mg of a faintly bluish fluffly powder. Analytical HPLC of this material gave a single peak.

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25

30



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Tolman, Richard L  
Marburg, Stephen  
Leanza, William J  
Lombardo, Victoria K

10

(ii) TITLE OF INVENTION: Peptide-Protein  
Conjugate Vaccines  
Having Anionic Spacers

15

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Merck & Co. Inc.  
(B) STREET: 126 Lincoln Avenue  
(C) CITY: Rahway  
(D) STATE: New Jersey  
(E) COUNTRY: United States of America  
(F) ZIP: 07065

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0,

25

Version #1.25

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

5

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Bencen, Gerard H

(B) REGISTRATION NUMBER: 35,746

(C) REFERENCE/DOCKET NUMBER: Case 18598

10

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (908) 594-3901

(B) TELEFAX: (908) 594-4720

15

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

20

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

25

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Pro Gly Arg

1

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

10

(B) TYPE: amino acid

(D) TOPOLOGY: both

## (ii) MOLECULE TYPE: peptide

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## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (v) FRAGMENT TYPE: internal

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## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

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(D) OTHER INFORMATION: /label= Nle

/note= "norleucine"

## (ix) FEATURE:

(A) NAME/KEY: Cross-links

(B) LOCATION: 2..9

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(D) OTHER INFORMATION: /label= cycle

/note= "amide bond through Lys  
epsilon amino"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Lys His Ile Gly Pro Gly Arg Ala

1

5

5

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

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(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /label= Nle

/note= "norleucine"

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## (ix) FEATURE:

(A) NAME/KEY: Cross-links

(B) LOCATION: 2..12

(D) OTHER INFORMATION: /label= cyclè

/note= "amide bond through Lys alpha

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amino"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 3

(D) OTHER INFORMATION: /label= Acap

/note= "6-aminocaproic acid"

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## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 12

(D) OTHER INFORMATION: /label= Acap

/note= "6-aminocaproic acid"

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Leu Lys Xaa His Ile Gly Pro Gly Arg Ala Phe Xaa

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5

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## (2) INFORMATION FOR SEQ ID NO:4:

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## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

25

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 2
- (D) OTHER INFORMATION: /label= Abu  
/note= "gamma amino butyric acid"

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## (ix) FEATURE:

- (A) NAME/KEY: Cross-links
- (B) LOCATION: 1..11
- (D) OTHER INFORMATION: /label= cycle  
/note= "amide through Lys epsilon amino"

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Xaa His Ile Ile Gly Pro Gly Arg Ala Phe  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: NO

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## (v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Cross-links
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= cycle  
/note= "amide through Lys epsilon amino"

25

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 2
- (D) OTHER INFORMATION: /label= Abu  
/note= "gamma amino butyric acid"

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Xaa Ser Ile Arg Ile Gly Pro Gly Arg Ala Phe Val Thr Ile Gly  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

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## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /label= N1e  
/note= "norleucine"

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## (ix) FEATURE:

(A) NAME/KEY: Disulfide-bond

(B) LOCATION: 2..21

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Lys	Cys	Tyr	Asn	Lys	Arg	Lys	Arg	Ile	His	Ile	Gly	Pro	Gly	Arg	Ala
1				5				10						15	

Phe	Tyr	Thr	Thr	Cys
				20

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

25

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

30

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= Nle  
/note= "norleucine"

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## (ix) FEATURE:

- (A) NAME/KEY: Disulfide-bond
- (B) LOCATION: 2..26

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Leu Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala  
1 5 10 15

Phe Tyr Thr Thr Lys Asn Ile Ile Gly Cys  
20 25

## (2) INFORMATION FOR SEQ ID NO:8:

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: NO

20

## (v) FRAGMENT TYPE: internal

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= Nle  
/note= "norleucine"

25

## (ix) FEATURE:

- (A) NAME/KEY: Cross-links
- (B) LOCATION: 2..10
- (D) OTHER INFORMATION: /label= cycle  
/note= "amide through Lys epsilon amino"

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Lys His Ile Gly Pro Gly Arg Ala Phe  
1 5 10



## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /label= Nle  
/note= "norleucine"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 3

(D) OTHER INFORMATION: /label= Acap  
/note= "6-aminocaproic acid"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 12

(D) OTHER INFORMATION: /label= Acap  
/note= "6-aminocaproic acid"

## (ix) FEATURE:

(A) NAME/KEY: Cross-links

(B) LOCATION: 2..12

(D) OTHER INFORMATION: /label= cycle  
/note= "amide through Lys alpha amino"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu	Lys	Xaa	Gln	Arg	Gly	Pro	Gly	Arg	Ala	Phe	Xaa
1				5					10		

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 13 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: both

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

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- (ix) FEATURE:  
    (A) NAME/KEY: Modified-site  
    (B) LOCATION: 1  
    (D) OTHER INFORMATION: /label= Nle  
        /note= "norleucine"

15

- (ix) FEATURE:  
    (A) NAME/KEY: Disulfide-bond  
    (B) LOCATION: 2..13

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Cys Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Cys  
1                    5                    10

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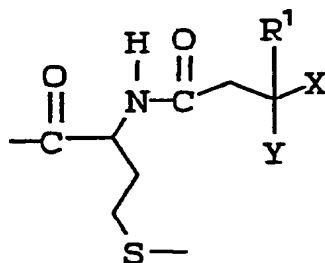
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WHAT IS CLAIMED IS:

1. A conjugate immunogen comprising the OMPC of Neisseria meningitidis b as a protein carrier, a principal neutralizing determinant, PND, of the human immunodeficiency virus, HIV as a peptidyl epitope against which immune responses are desired, and a low molecular weight anionic spacer linking the protein carrier to the peptidyl epitope.

2. The conjugate of Claim 1 wherein the PND comprises the sequence Gly Pro Gly Arg [Sequence. Id:1:].

3. The conjugate of Claim 2 wherein the conjugate comprises a spacer of formula:



wherein the free carbonyl on the left is bonded to the OMPC and the sulfur is bonded to the HIV PND;  
R<sup>1</sup> is hydrogen or a lower alkyl of 1 to 4 carbons;

(i) X is:

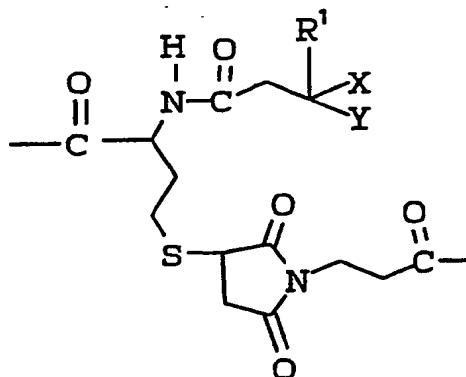
- (a)  $-\text{PO}_3^-$ ,
- (b)  $-\text{SO}_3^-$ , or
- (c)  $-\text{CO}_2\text{H}$ ; and

Y is hydrogen; or

(ii) both X and Y are  $-\text{CH}_2\text{CO}_2\text{H}$ , or

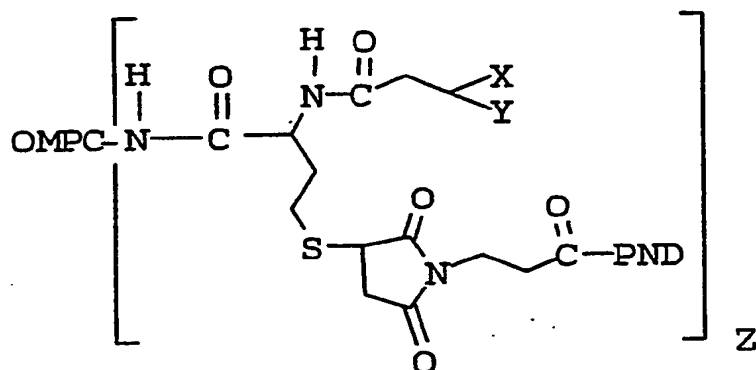
(iii) X is  $\text{CO}_2\text{H}$  and Y is  $-\text{CH}_2\text{CO}_2\text{H}$ .

4. The conjugate of Claim 3 wherein the conjugate comprises a spacer of formula:



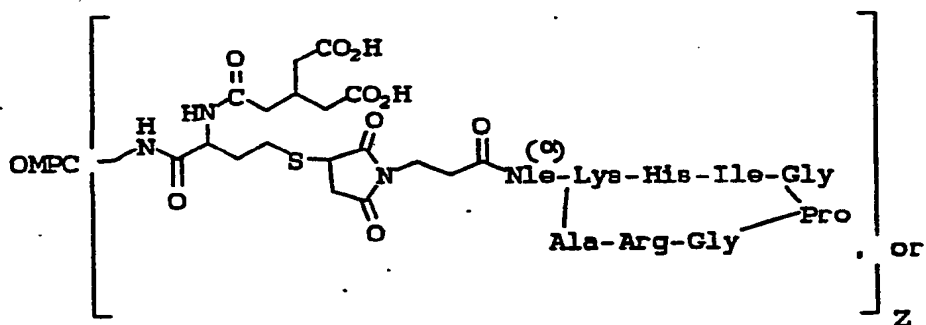
wherein the free carbonyl on the left is bonded to the OMPC and the free carbonyl on the right is bonded to the HIV PND; all other variables are as defined in Claim 3.

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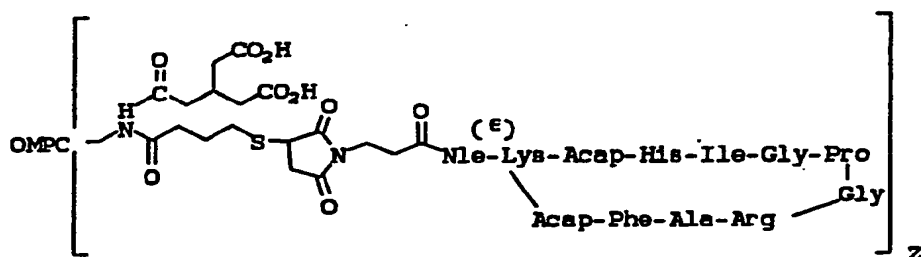
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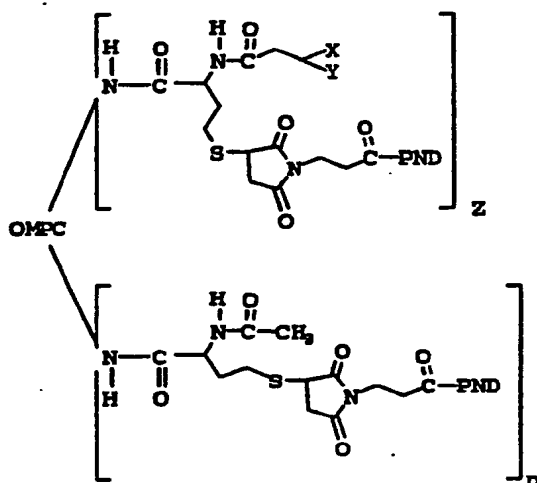
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(b) (Seq. Id:3:)



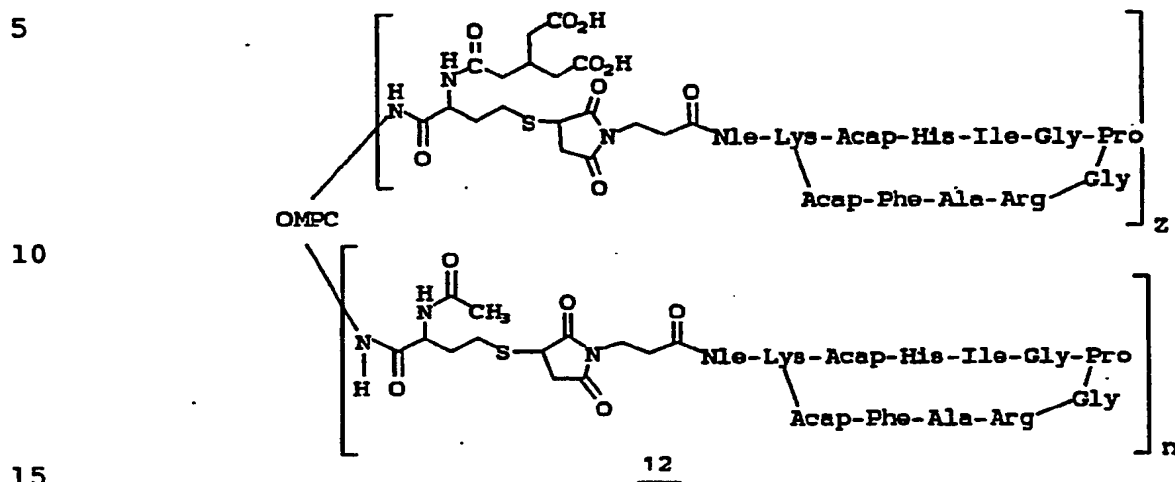
7. The conjugate of Claim 4 comprising a neutral spacer in addition to the anionic spacer.

15 8. The conjugate of Claim 7 having the formula:



30 wherein  $1 \leq Z \leq 4000$ , and  $1 \leq n \leq 3000$ .

9. The conjugate of Claim 8 having the formula, (Seq. Id:3:)



10. A conjugate of any of Claims 1-5, 7, or 8 wherein the peptide is:

cPND502 SEQ. ID:4

Lys Abu His Ile Ile Gly Pro Gly Arg Ala Phe ;

cPND535 SEQ. ID:5

Lys Abu Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala  
Phe Val Thr Ile Gly ;

cPND078 SEQ. ID:6

Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Cys ; or

cPND402 SEQ. ID:7

Nle Cys Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile  
Gly Cys.

11. A composition comprising an inert carrier and an immunologically effective amount of the conjugate of any of Claims 1-10.

5 12. A method for inducing anti-peptide, anti-HIV or HIV neutralizing immune responses in a mammal which comprises administering the composition of Claim 11.

13. A process for making a conjugate immunogen which comprises:

10 a) Thiolating the OMPC of Neisseria meningitidis b with an anionic thiolactone;

b) Suspending the thiolated OMPC of step a) in a buffer containing about 0.5 % deoxycholate;

15 c) Maleimidating or bromoacetylating an HIV PND peptide;

d) Reacting the maleimidated or bromoacetylated peptide of step c) with the thiolated OMPC of step b); and

e) Isolating the conjugate from the reaction of step d).

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**Relevant Technical Fields**

(i) UK Cl (Ed.L) C3H (HHX2)

(ii) Int Cl (Ed.5) C07K 17/06; A61K 39/385

Search Examiner  
 C SHERRINGTON

Date of completion of Search  
 4 JANUARY 1994

**Databases (see below)**

(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE DATABASES: WPI, DIALOG/BIOTECH

Documents considered relevant following a search in respect of Claims :-  
 1-11, 13

**Categories of documents**

- |  |   |
|--|---|
| <p><b>X:</b> Document indicating lack of novelty or of inventive step.</p> <p><b>Y:</b> Document indicating lack of inventive step if combined with one or more other documents of the same category.</p> <p><b>A:</b> Document indicating technological background and/or state of the art.</p> | <p><b>P:</b> Document published on or after the declared priority date but before the filing date of the present application.</p> <p><b>E:</b> Patent document published on or after, but with priority date earlier than, the filing date of the present application.</p> <p><b>&amp;:</b> Member of the same patent family; corresponding document.</p> |
|--|---|

Category	Identity of document and relevant passages		Relevant to claim(s)
X	EP 0467700 A2	(MERCK & CO INC) whole document, especially page 6, line 56 to page 10, line 19; Claims 9,13	1,2
X	EP 0467714 A1	(MERCK & CO INC) especially page 14, line 46 to page 16, line 58	1,2
X	EP 0468714 A2	(MERCK & CO INC) whole document	1,2
P,X	EP 0519554 A1	(MERCK & CO INC) whole document, especially page 8, line 29 to page 11, line 1, Claims 6 to 10	1,2

**Databases:** The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).